EXHIBIT 3

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Phenotypic and Functional Comparison of Cultures of Marrow-Derived Mesenchymal Stem Cells (MSCs) and Stromal Cells

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Mesenchymal stem cells (MSCs) are a population of pluripotent cells within the bone marrow microenvironment defined by their ability to differentiate into cells of the osteogenic, chondrogenic, tendonogenic, adipogenic, and myogenic lineages. We have developed methodologies to isolate and culture-expand MSCs from human bone marrow, and in this study, we examined the MSC's role as a stromal cell precursor capable of supporting hematopoietic differentiation in vitro. We examined the morphology, phenotype, and in vitro function of cultures of MSCs and traditional marrow-derived stromal cells (MDSCs) from the same marrow sample. MSCs are morphologically distinct from MDSC cultures, and flow cytometric analyses show that MSCs are a homogeneous cell population devoid of hematopoietic cells. RT-PCR analysis of cytokine and growth factor mRNA in MSCs and MDSCs revealed a very similar pattern of mRNAs including IL-6, -7, -8, -11, -12, -14, and -15, M-CSF, Flt-3 ligand, and SCF. Steady-state levels of IL-11 and IL-12 mRNA were found to be greater in MSCs. Addition of IL-1α induced steady-state levels of G-CSF and GM-CSF mRNA in both cell preparations. In contrast, IL-1α induced IL-1α and LIF mRNA levels only in MSCs, further emphasizing phenotypic differences between MSCs and MDSCs. In long-term bone marrow culture (LTBMC), MSCs maintained the hematopoietic differentiation of $CD34^+$ hematopoietic progenitor cells. Together, these data suggest that MSCs represent an important cellular component of the bone marrow microenvironment. J. Cell. Physiol. 176:57–66, 1998. © 1998 Wiley-Liss, Inc.

The bone marrow is comprised of hematopoietic cells and adherent stromal cells of nonhematopoietic origin which together with the extracellular matrix provide a supportive scaffolding termed the bone marrow microenvironment. The cellular components of the marrow microenvironment include reticular endothelial cells, macrophages, adipocytes, fibroblasts, and osteogenic precursor cells (Beresfords, 1989; Dorshkind, 1990). By promoting cell-to-cell interactions (Williams, 1994), the expression and presentation of cytokines and growth factors, and the secretion of extracellular matrix proteins, the marrow microenvironment provides a favorable platform for the localization, self-renewal, and differentiation of hematopoietic stem cells (HSCs).

Histological analysis of the hematopoietic bone marrow indicates the presence of a complex array of niches where specific cell types play distinct functions in the regulation of hematopoiesis (Weiss, 1995). Although numerous studies with mixed stromal cell cultures have advanced our understanding of the role of the bone marrow microenvironment in hematopoiesis, detailed molecular and functional characterization of the individual cellular components of the stroma is incomplete. Immortalized bone marrow stromal cells (Dorshkind and Landreth, 1992; Deryugina and Muller-Sieburg, 1993; Roecklein and Torok-Storb, 1995; Wineman et al., 1993; Mosca et al., 1995) have been used in long-term bone marrow cultures (LTBMC) to further define the heterogeneity in the marrow microenvironment (Dexter et al., 1977). The major disadvantages of relying on transformed and immortalized cell lines to determine the functional elements of the marrow microenvironment lies in the potential of these cells to undergo morphologic, phenotypic, and regulatory changes that make them unpredictable surrogates for their normal cell counterparts.

In addition to hematopoietic stem cells, the bone mar-

Abbreviations: BMM bone marrow microenvironment; HSC hematopoietic stem cell; LTBMC long-term bone marrow culture; LTC-IC long-term culture-initiating cell; MDSC marrow-derived stromal cells; MSC mesenchymal stem cell.

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row microenvironment is also home to pluripotent nonhematopoietic precursor cells that have the ability to form specific connective tissues such as bone, cartilage, and the marrow stroma (Beresfords, 1989; Dorshkind, 1990; Caplan, 1991). These pluripotent precursors, termed mesenchymal stem cells (MSCs), have been shown to differentiate into various cell lineages, including osteoblasts (Haynesworth et al., 1992a), chondrocytes (Johnstone et al., 1996), and adipocytes (Pittenger et. al., 1996) when placed in appropriate in vitro and in vivo environments. Previous studies have also shown that MSCs secrete a number of cytokines such as interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), and granulocyte-macrophage colony stimulating factor (GM-CSF) (Haynesworth et al., 1996). In this study, we examined the expression of cell surface molecules, cytokines, and growth factors by MSCs and test their ability to maintain hematopoiesis in LTBMC with $CD34^+$ hematopoietic progenitors. The analysis of MSCs was compared to MDSCs prepared from the same marrow sample.

MATERIALS AND METHODS

Isolation and culture expansion of MSCs and MDSCs

Bone marrow samples were collected from healthy human donors at the Johns Hopkins University under an Institutional Review Board-approved protocol. MSCs were isolated and cultured according to modifications of a previously reported method (Haynesworth et al., 1992a). Briefly, 25 ml of heparinized bone marrow was mixed with an equal volume of phosphate-buffered saline (PBS) (Life Technologies, Gaithersburg, MD) and centrifuged at 900g for 10 min at room temperature. Washed cells were resuspended in PBS to a final density of 4×10^7 cells/ml, and a 5 ml aliquot was layered over a 1.073 g/ml Percoll solution (Pharmacia, Piscataway, NJ) and centrifuged at 900g for 30 min. Mononuclear cells collecting at the interface were recovered, resuspended in human MSC medium, and plated at a density of 3×10^7 cells per 185 cm^2 Nunclon Solo flask (Nunc Inc., Naperville, IL). Human MSC medium consisted of Dulbecco's Modified Eagles Medium-Low Glucose (DMEM-LG) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Biocell Laboratories, Rancho Dominquez, CA) and 1% antibiotic-antimycotic solution (Life Technologies). The FBS used in MSC medium was selected based on its ability to maximize recovery and culture expansion of MSCs from bone marrow (Lennon et al., 1996). MSC cultures were maintained at 37°C in 5% CO_2 in air, with medium changes after 48 h and every 3–4 days thereafter. When the cultures reached 90% of confluence, cells were recovered by the addition of a solution containing 0.25% trypsin-EDTA (Life Technologies) and replated at a density of 1×10^6 cells per 185 cm^2 flask as passage 1 cells.

MDSCs were isolated from the same bone marrow sample using the following procedure. Bone marrow was mixed with an equal volume of PBS containing 2% bovine serum albumin (BSA) (Life Technologies), 0.6% sodium citrate (Sigma, St. Louis, MO), and 1% penicillin-streptomycin (Life Technologies), and aliquots were layered over Ficoll-Paque (1.077 gm/ml) (Pharmacia) and centrifuged at 800g for 20 min. The mononuclear cells that collected at the interface were suspended in LTBMC medium and plated at a density of 60×10^6 cells per 185 cm^2 flask and incubated at 37°C in 5% CO_2 in air. LTBMC medium consisted of MyeloCult H5100 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 1 μM hydrocortisone (Sigma). MyeloCult H5100 is made up of 12.5% FBS, 12.5% horse serum, 0.2 mM i-inositol, 20 mM folic acid, 0.1 mM 2-Mercaptoethanol, 2 mM L-glutamine in Alpha MEM. Medium was changed every 7 days, and primary cultures were recovered by trypsinization when the cells reached 90% of confluence and replated at a density of 1×10^6 cells per 185 cm^2 flask as passage 1 cells.

Flow cytometry

Analysis of cell surface molecules was performed on passage 1 cultures of MSCs and MDSCs using flow cytometry and the following procedure. Media was removed from flasks, and cell layers were washed twice with PBS and detached from the flask by incubation with a solution of 0.25% trypsin-EDTA for 5 min at room temperature. Cells were recovered by centrifugation and washed in flow cytometry buffer consisting of 2% BSA and 0.1% sodium azide (Sigma) in PBS. Aliquots (2×10^5 cells) were incubated with conjugated monoclonal antibodies, either SH-2-PE (Haynesworth et al., 1992b) and SB-10-FITC (Bruder et al., 1997) (Osiris Therapeutics Inc., Baltimore, MD) or anti-CD14-PE and anti-CD45-Cy-chrome (PharMingen, San Diego, CA). All incubations with antibodies were performed for 20 min, after which cells were washed with flow cytometry buffer. Washed cells were pelleted and resuspended in flow cytometry buffer containing 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Nonspecific fluorescence was determined using equal aliquots of the cell preparation that were incubated with anti-mouse monoclonal antibodies (PharMingen). Data for both the MSCs and the MDSCs cultures were collected under identical parameters and analyzed by collecting 10,000 events on a Becton Dickinson Vantage instrument (San Jose, CA) using CellQuest software.

Long-term bone marrow cultures (LTBMC)

To establish LTBMC, we removed primary cultures of MSCs and MDSCs from the tissue culture flask by trypsinization. Cells were suspended at a density of 2×10^6 cells/ml in LTBMC medium and exposed to 16 Gy γ irradiation from a ^{137}Cs source. The irradiated cells were then plated in triplicate at a density of $3–5 \times 10^5$ cells per well in a six-well culture plate, and cultures were incubated overnight.

$CD34^+$ cells were isolated from the Ficoll (1.077 gm/ml) fraction of fresh bone marrow using the Dynal CD34 progenitor cell selection system (Dynal Inc., Lake Success, NY) according to the manufacturer's protocol. Generally, the total number of $CD34^+$ cells recovered were 1–2% of the total mononuclear cell fraction. Aliquots (1×10^5) $CD34^+$ cells were layered onto each well of either MSC or MDSCs, and the cocultures were incubated at 33°C in 5% CO_2 in air in LTBMC medium. Half of the medium was replaced weekly with fresh medium for 5 weeks. Experimental controls consisted of LTBMC of $CD34^+$ cells in the absence of preformed adherent monolayer cells.

After 5 weeks of coculture, nonadherent cells from the LTBMC were collected from the medium by centrifugation, and the adherent cells from each well were recovered by trypsinization. Cells from each fraction were resuspended in 0.3 ml of LTBMC medium, and the suspension was added to 2.7 ml of methylcellulose medium, MethoCult 4435 (Stem Cell Technologies). Aliquots (1 ml) of the cell mixture were plated in duplicate in 35 mm Nunc dishes (Nunc Inc.) and incubated at 37°C in 5% CO_2 in air. After 2 weeks, colonies composed of >50 cells were scored, and the numbers from both fractions were combined and used for statistical analysis.

RNA preparation and analysis

Passage 1 MSCs were incubated for 24 h in either MSC medium, MSC medium containing 10 units/ml IL-1α (Boehringer Mannheim, Indianapolis, IN), or LTBMC medium. Passage 1 MDSCs were maintained in either LTBMC medium or in LTBMC medium in which 10 units/ml IL-1α was added for 24 h. Total RNA was extracted from cultures of MSCs and MDSCs by modification of the method of Chirgwin et al. (1979). Briefly, cells were lysed in a solution consisting of 4 M guanidinium isothiocynate (Sigma), 0.03 M sodium acetate (Sigma), and 0.4 g/ml of cesium chloride (Life Technologies). Lysates were layered over 3 ml of 5.7 M CsCl and centrifuged for 18 h at 155,000g in a Beckman (Palo Alto, CA) SW40 rotor. RNA was dissolved in diethyl pyrocarbonate-(DEPC) (Sigma) treated water and precipitated by the addition of 1/10 volume of 0.3 M sodium acetate and 2 volumes of absolute ethanol. RNA was recovered by centrifugation and dissolved in DEPC-treated water at a concentration of 0.5 mg/ml.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using total RNA as a template, a RNA PCR core kit (Perkin-Elmer Cetus, Norfolk, CT), and the oligonucleotide primers (Operon, Alameda, CA) in Table 1. The RT-PCR products were analyzed by electrophoresis on a 2% agarose gel containing 1 μg/ml ethidium bromide.

For analysis of gene expression, we utilized the PCR ELISA kit (Boehringer Mannheim) and the procedure recommended by the manufacturer. Briefly, PCR was performed in the presence of digoxigenin-labeled nucleotides to label the amplified product. The PCR product (25 μl) was denatured and allowed to hybridize in solution to a 5′-biotinylated oligonucleotide probe at 37°C in a streptavidin-coated microtiter plate. The bound probe-PCR product was detected by an anti-digoxigenin peroxidase conjugate and by the use of the colorimetric substrate ABTS. In comparison to the β-2 microglobulin standard, we evaluated the presence of amplified product from each PCR reaction.

Statistical analysis

Results from experiments obtained from multiple experiments were reported as the mean ± standard error of the mean (SEM). Significance levels were determined by two-sided Student's *t*-test analysis.

RESULTS

MSCs are a homogenous population of adherent bone marrow cells with a distinct morphology and cell surface protein expression

We sought to establish the morphological differences between MSCs and MDSCs derived from the same bone marrow sample. MDSCs were cultured from the Ficoll fraction of mononuclear cells from marrow in an enriched medium containing 25% serum with 1 μM hydrocortisone. Primary and passage 1 cultures of MDSCs contain a heterogeneous population of hematopoietic and stromal cells (Fig. 1). After 14 days, primary cultures of MDSCs show evidence of hematopoiesis, as indicated by formation of cobblestone areas formation. MSCs were isolated from the Percoll fraction of mononuclear cells and cultured in a medium containing 10% FBS. In contrast to the MDSCs, primary and passage 1 cultures of MSCs appear morphologically as a homogenous population of fibroblastoid cells (Fig. 1). The results demonstrate that the isolation and culture conditions established for MSCs select a distinct population of bone marrow-derived adherent cells.

To compare the expression of cell surface molecules on MSCs and MDSCs obtained from the same donor, we performed flow cytometric analyses. We collected cells from passage 1 cultures and labeled them with monoclonal antibodies, SH-2 and SB-10, that recognize antigens present on the MSCs as well as monoclonal antibodies that recognize antigens present on hematopoietic cells. The identity of SH-2 is being pursued, while SB-10 has been identified as ALCAM (activated leucocyte-cell adhesion molecule) (Bruder et al., in press). The results show that MSCs are a homogenous population of cells that express antigens recognized by SH-2 and SB-10 and the absence of hematopoietic cells. The antigens SH-2 and SB-10 are also present on cells that constitute MDSC cultures (Fig. 2). In contrast to MSCs, the MDSCs were positive for cells expressing both CD14 and CD45 cell surface antigens, demonstrating the presence of hematopoietic cells in these cultures. $CD34^+$ cells were undetected in both passage 1 cultures of MSCs and MDSCs (data not shown). These data emphasize the homogenous characteristic of MSC cultures and indicate the presence of MSC antigens on cells of MDSC cultures.

MSCs show similar cytokine and growth factor expression as MDSCs

We used RT-PCR to compare the steady-state levels of mRNAs for various cytokine and growth factors in the MSCs and MDSCs prepared from the bone marrow cells of the same donor (Fig. 3). We included the amplification of β2-microglobulin mRNA in the analysis in order to monitor equal input of RNA into the reactions. Our initial analysis performed on MSCs included evaluation of mRNA for IL-1 to IL-4, IL-6 to IL-8, and IL-10 to IL-15, LIF, granulocyte colony stimulating factor (G-CSF), GM-CSF, macrophage colony stimulating factor M-CSF, Flt-3 ligand, and stem cell factor (SCF). We were unable to detect mRNAs for IL-2, -3, -4, -10, and -13 in MSCs, although our oligonucleotide primer pairs amplified the correct molecules from appropriate cell lines. For positive PCR signals, we confirmed the amplification products with 5′-biotinylated oligonucleotide primer probes using the PCR-ELISA procedure. The analysis shown in Figure 3 includes only those mRNAs found to be present in MSCs.

We found MSCs maintained in normal culture condition to constitutively express mRNAs for IL-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, LIF, M-CSF, Flt-3 ligand, and SCF (Fig. 3). These results corroborate previous

TABLE 1. Oligonucleotide primers used for the PCR detection of gene transcripts

Oligonucleotide primers/5′-biotinylated probes	Size (bp)	References/accession #
Hu Beta-2 microblobulin 5′ TCTGGCCTTGAGGCTATCCAGCGT 3′ GTGGTTCACACGGCAGGCATACTC Probe 5′-biotinylated CATCCATCCGACATTGAAGTTGAC	270	Kollman et al. (1994)
Hu IL-1 alpha 5′ ATGGCCAAAGTTCCAGACATGTTTG 3′ GGTTTTCCAGTATCTGAAAGTCAGT Probe 5′-biotinylated TCTGTGTCTCTGAGTATCTCTG	808	X02531
Hu IL-1 beta 5′ CTTCATCTTTTGAAGAAGAACCTATCTTCTT 3′ AATTTTTGGGATCTACACTCTCCAGCTGTA Probe 5′-biotinylated AGTGGTGTTCTCCATGTCCTT	331	M15330
Hu IL-6 5′ GTAGCCGCCCCACACAGACAGCC 3′ GCCATCTTTGGAAGGTTCAGG Probe 5′-biotinylated ATCTCAGCCCTGAGAAAGGAG	174	Auffray et al. (1994)
Hu IL-7 5′ ATGTTCCATGTTTCTTTTAGGTATATCT 3′ TGCATTTCTCAAATGCCCTAATCCG Probe 5′-biotinylated AAGAAAACCAGCTGCCCTGGGT	681	J04156
Hu IL-8 5′ TCTGCAGCTCTGTGTGAAGGT 3′ TGAATTCTCAGCCCTCTTCAA Probe 5′-biotinylated GATTGAGAGTGGACCACACTG	252	M28130
Hu IL-11 5′ ATGAACTGTGTTTGCCGCCTG 3′ GAGCTGTAGAGCTCCCAGTGC Probe 5′-biotinylated ACGGGGACCACAACCTGGATT	331	M81890
Hu IL-12 (p40) 5′ TCACAAAGGAGGCGAGGTTC 3′ TGAACGGCATCCACCATGAC Probe 5′-biotinylated GGTGGCTGACGACAATCAGTA	378	M65290
Hu IL-14 5′ GTGAATGATAAGGCTACTGAGAAG 3′ GAGCATTTCTCTGTCTGACTTTGAG Probe 5′-biotinylated CTGCTCACAAGACAAATGCAGT	368	L15344
Hu IL-15 5′ CACATTTGAGAAGTATTTCCATCCAGTGC 3′ GAAGACAAACTGTTGTTTGCTAGGATG Probe 5′-biotinylated CAATCTATGCATATTGATGCTAC	356	X94223
Hu LIF 5′ AACAACCTCATGAACCAGATCAGGAGC 3′ ATCCTTACCCGAGGTGTCAGGGCCGTAGG Probe 5′-biotinylated CAACCTGGACAAGCTATGTGG	405	M63420
Hu G-CSF 5′ AGCTTCCTGCTCAAGTGCTTAGAG 3′ TTCTTCCATCTGCTGCCAGATGGT Probe 5′-biotinylated CACCTACAAGCTGTGCCACC	346	E08531
Hu GM-CSF 5′ GTCTCCTGAACCTGAGTAGAGACA 3′ AAGGGGATGACAAGCAGAAAGTCC Probe 5′-biotinylated ATGGCCAGCCACTACAAGCAG	286	M13207
Hu M-CSF 5′ TTGGGAGTGGACACCTGCAGTCT 3′ CCTTGGTGAAGCAGCTCTTCAGCC Probe 5′-biotinylated TAATGGAGGACACCATGCGCT	248	Kollman et al. (1994)
Hu Flt-3 ligand 5′ TGGAGCCCAACAACCTATCTC 3′ GGGCTGAAAGGCACATTTGGT Probe 5′-biotinylated TTCAAGATTACCCAGTCACCG	333	U03858
Hu SCF 5′ CTCCTATTTAATCCTCTCGTC 3′ TACTACCATCTCGCTTATCCA Probe 5′-biotinylated TAACCCTCAAATATGTCCCC	177	Auffray et al. (1994)

evidence for the presence of many of these cytokines in media conditioned by MSCs (Haynesworth et al., 1996). We found the MDSCs cultured in LTBMC medium to also express these mRNAs (Fig. 3). Under basal conditions, the mRNA for IL-1α and β, G-CSF, and GM-CSF were not detected in cultures of either MSCs or MDSCs. Interestingly, the major amplified product for IL-12 was not detected in MDSCs; however, a minor amplified product of smaller size was observed in both MSCs and MDSCs.

The induction of cytokine expression following exposure to inflammatory molecules such as IL-1 (Aman et al., 1994; Caldwell and Emerson, 1994) is a characteristic response of cultured bone marrow-derived stromal cells. Addition of IL-1α (1, 10, or 100 units/ml) for 4–24 h increased the steady-state level for several cyto-

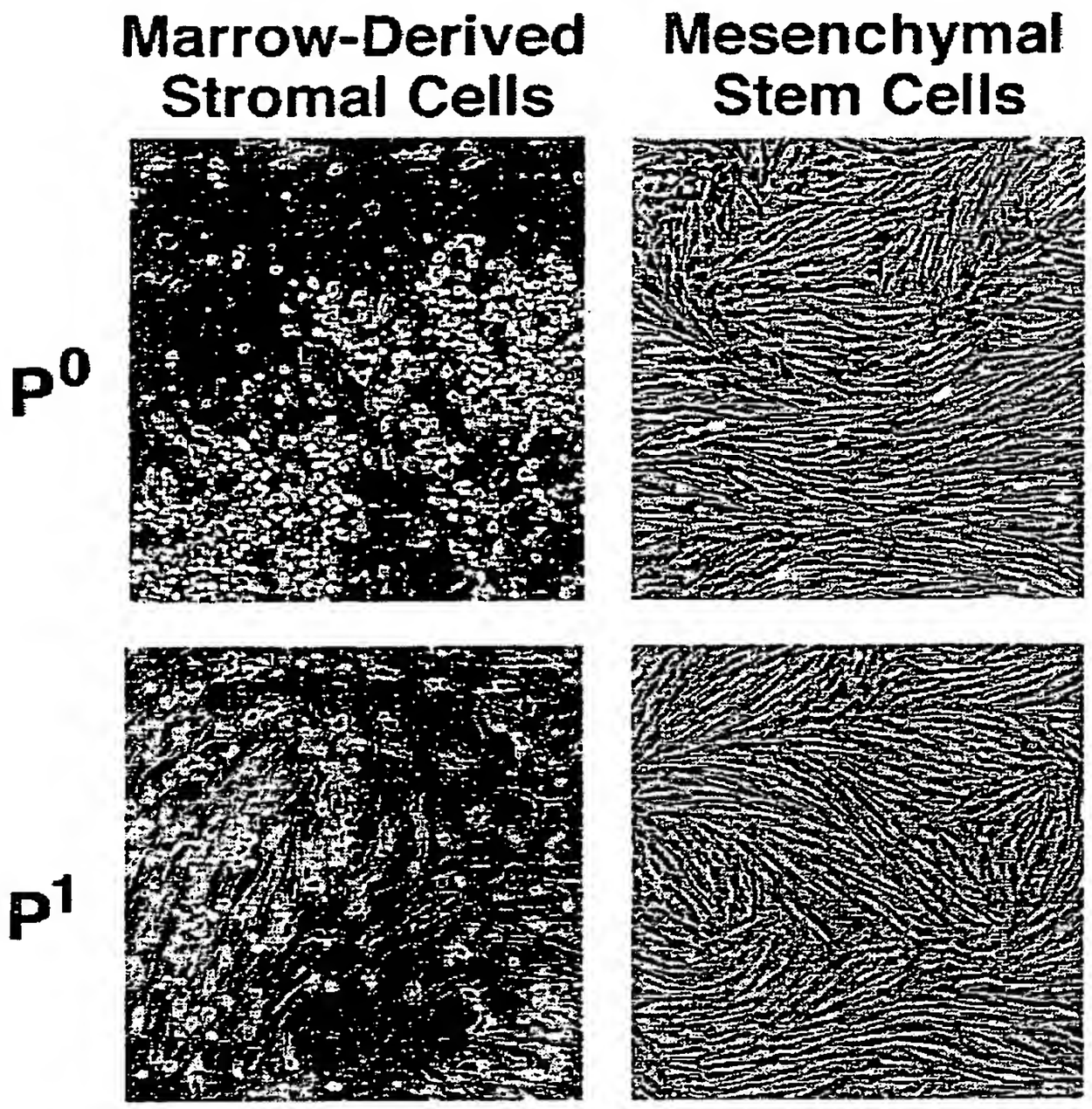


Fig. 1. Morphology of primary (P^0) and passage 1 (P^1) cultures of MDSCs and MSCs. MDSCs were isolated from bone marrow cells by Ficoll fractionation and plated in LTBMC medium. MSCs were isolated from bone marrow cells by Percoll fractionation and plated in MSC medium. P^0 cultures were maintained for 14 days, after which they were trypsinized and replated as P^1 cells. Cultures were maintained as P^1 cells in their respective medium. Photomicrographs are representative of P^0 and P^1 cultures as shown above. ×100.

kine and growth factor mRNAs (data not shown). In Figure 3, we show that a 24 h treatment of MSC cultures with 10 units/ml IL-1α increased the steady-state levels of IL-1α, IL-1β, IL-6, IL-8, IL-11, G-CSF, GM-CSF, and LIF mRNAs. MDSCs cultures treated with IL-1α showed a similar increase in the levels of some of these mRNAs; however, the relative levels of IL-1α, IL-1β, IL-8, IL-11, and LIF mRNA did not appear to change to the same degree in these cultures. IL-1α treatment seemed not to alter the expression of IL-7, IL-12, IL-14, IL-15, M-CSF, Flt-3 ligand, and SCF genes in either MSCs or MDSCs (Fig. 3, lanes 2 and 4). To further confirm the results, we analyzed the expression of IL-1 receptor mRNA on MSCs and on MDSCs and found it to be similar (data not shown), therefore suggesting a comparable effect of IL-1α on both MSCs and MDSCs. Since the composition of the medium used for LTBMC assays is markedly different than MSC media, we examined the effect that switching from MSC to LTBMC medium had on cytokine and growth factor mRNA expression in MSC cultures. Replacement of the MSC media with LTBMC media for 24 h did not alter the steady-state levels of most mRNAs examined with the exception of mRNA for IL-11, which appeared to be lower than those observed in cultures maintained in MSC medium.

We further analyzed the regulation of gene expression of IL-1α, G-CSF, GM-CSF, and LIF by IL-1α. The number of copies of gene transcripts for the individual cytokine and growth factor was determined by analyzing an equal amount of RNA from either untreated MSCs or IL-1α-treated MSCs in RT-PCR reactions, and quantitation of PCR amplified products was performed using 5′-biotinylated oligonucleotide primer probes specific for the genes and PCR-ELISA analysis. The number of gene copies for the cytokines and growth factors were calculated relative to the PCR amplification of known gene copies for the β-2 microglobulin gene. As seen in Table 2, the results not only confirm our observation seen in Figure 3 but also show that treatment of MSCs with IL-1α upregulates the expression of IL-1α, G-CSF, GM-CSF, and LIF by 50-, 800-, 80-, and 350-fold, respectively.

MSCs support hematopoietic differentiation in vitro

The expression of these cytokines and growth factors suggests that MSCs may function in hematopoiesis. Therefore, LTBMCs were established with irradiated MSCs or MDSCs derived from the same marrow sample. Coculture of MDSCs or MSCs with allogeneic immuno-selected $CD34^+$ hematopoietic progenitor cells resulted in the formation of cobblestone areas representative of hematopoietic progenitor cell proliferation and differentiation (data not shown). Cells derived from LTBMC were plated after 5 weeks in media containing

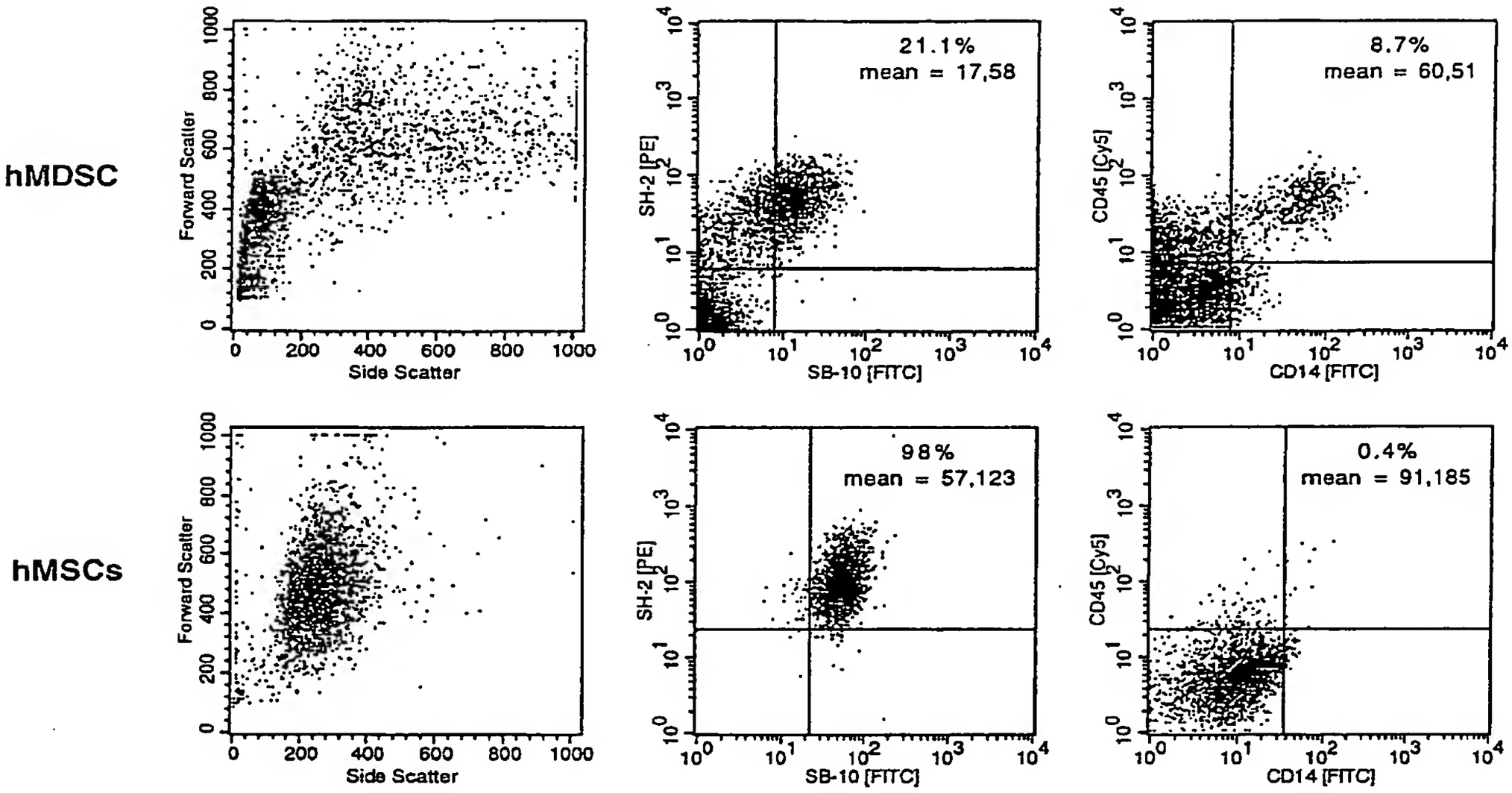


Fig. 2. Flow cytometric analysis of cultured MDSCs and MSCs with monoclonal antibodies. Primary cultures of MDSCs and MSCs were expanded to passage 1 in LTBMC medium and MSC medium, respectively. Populations of these cells were stained with monoclonal antibodies SH-2, SB-10, CD14, and CD45 and analyzed by flow cytometry. The percent (%) and mean fluorescence intensity on the axis were determined by cells in the upper right quadrant. These data are representative of analysis performed on three individual MSC and MDSC cultures.

methylcellulose to assess hematopoietic colony formation. As seen in Figure 4, MSCs from four individual marrow donors maintained long-term culture initiating cells (LTC-IC) in this assay. MSCs from three of four donors were less efficient at maintaining hematopoietic progenitors than the corresponding MDSCs, suggesting some donor variability in this cellular function. This donor variability was particularly evident in the results of assays performed with MSCs from donors 100 and 101. Although these MSCs were cocultured with aliquots of the same $CD34^+$ cell preparation, there was a large difference in the number of hematopoietic colonies that formed in methylcellulose. Experimental controls of LTBMC of $CD34^+$ cells in the absence of adherent monolayer cells did not result in any colony formation. These data demonstrate that cultures of MSCs as a homogenous population have the ability to maintain LTC-IC in hematopoietic long-term culture assay.

DISCUSSION

The bone marrow microenvironment is a complex cellular structure. Investigators have employed primary cells as well as stromal cell lines both from the murine and human bone marrow (Dorshkind and Landreth, 1992; Deryugina and Muller-Sieburg, 1993; Wineman et al., 1993; Roecklein and Torok-Storb, 1995; Mosca et al., 1995) to attain insight towards understanding the cellular and molecular components of the microenvironment and their regulation and maintenance of hematopoiesis. The human stromal cell lines have been established from the LTBMC system (Dexter et al., 1977), while most of the murine cell lines were established by the bone marrow culture systems as described by Dexter et al. (1977) and later modified by Greenberger (1978). These stromal cell lines in general are functionally heterogenous with respect to their ability to sustain B lineage and myeloid cells (Deryugina and Muller-Sieburg, 1993). The murine Whitlock-Witte LTBMC (Whitlock and Witte, 1982) have been crucial for the analysis of the events that regulate proliferation of B lineage cells. Attempts to establish similar human equivalent cell lines have not been successful. A comprehensive analysis (Wineman et al., 1996) designed to assess heterogeneity within the murine stromal compartment was performed which lent experimental support to the hypothesis that the transition from primitive to less primitive stem cells is regulated by distinct stromal cell niches that interact with specific subsets of stem cells. Since similar experiments related to dissecting out the human bone marrow microenvironment are difficult to perform, our understanding has been limited to study of cell lines and to a lesser extent primary cells derived from bone marrow.

Although these studies have increased our knowledge of the bone marrow nonhematopoietic compartment, the precise identity of different cellular compo-

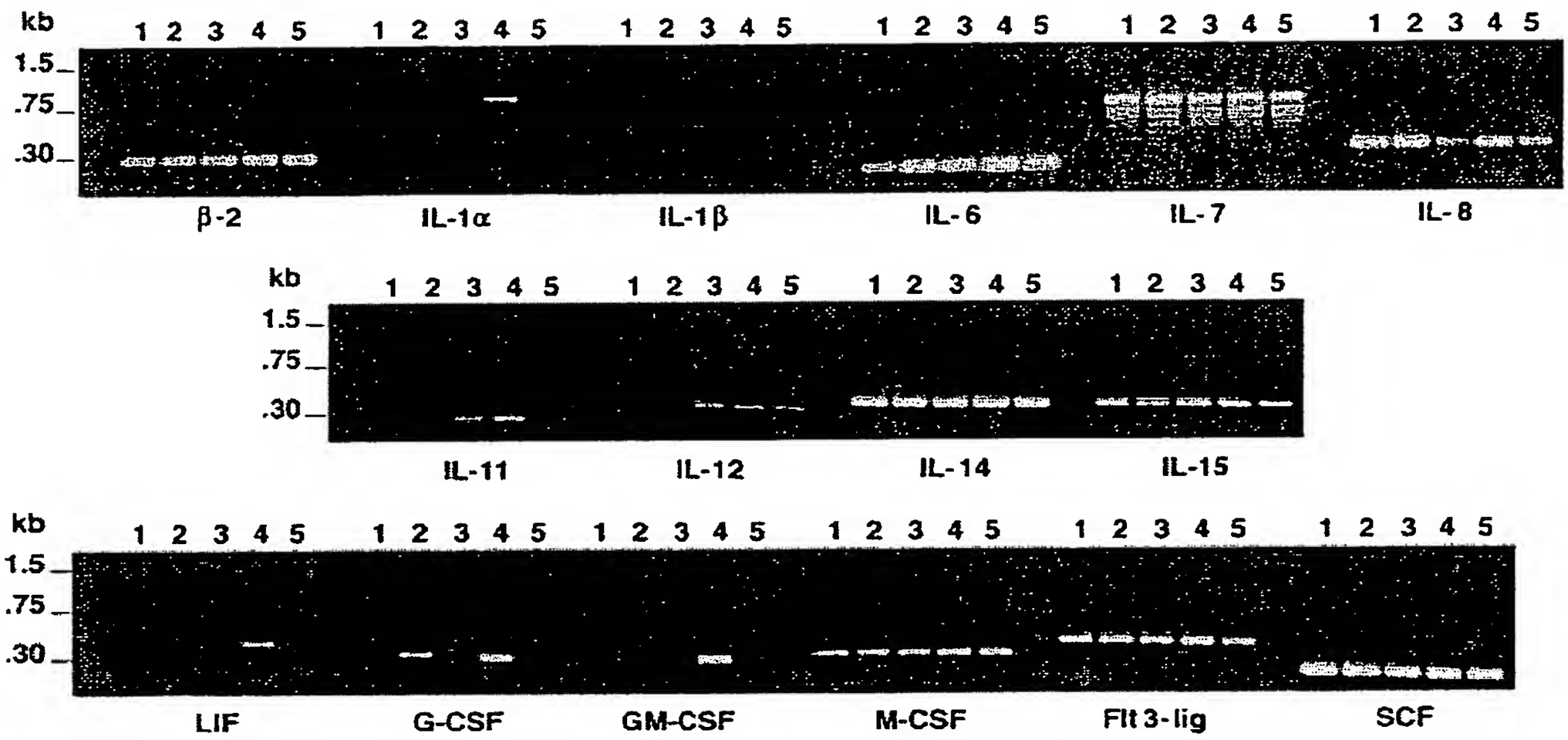


Fig. 3. Gene expression of cytokines and growth factors by MDSCs and MSCs. Total RNA was isolated from MDSCs cultured in LTBMC medium (lanes 1) and LTBMC medium with IL-1α (10 U/ml) for 24 h (lanes 2). Total RNA was also isolated from MSCs cultured in MSC medium (lanes 3), in MSC medium with IL-1α (10 U/ml) for 24 h (lanes 4), and in LTBMC medium for 24 h (lanes 5). Total RNA samples were used for synthesis of cDNA, which was then amplified for 35 cycles using specific primers for cytokines and growth factors genes. Amplified products were analyzed on 2% agarose gels and stained with ethidium bromide. To monitor equal input of RNA in the reverse transcriptase reactions, we used β2-microglobulin gene amplification. Molecular weight markers are represented (kb). These data are representative of results obtained from cultures preapared from at least three individual marrow donors.

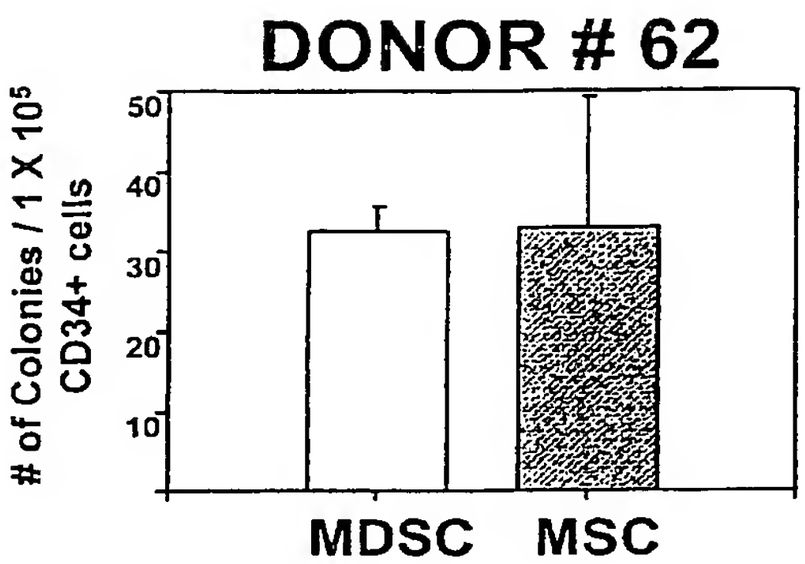
TABLE 2. Comparison of regulation of mRNA expression in IL-1-treated MSCs by PCR-ELISA analysis[1]

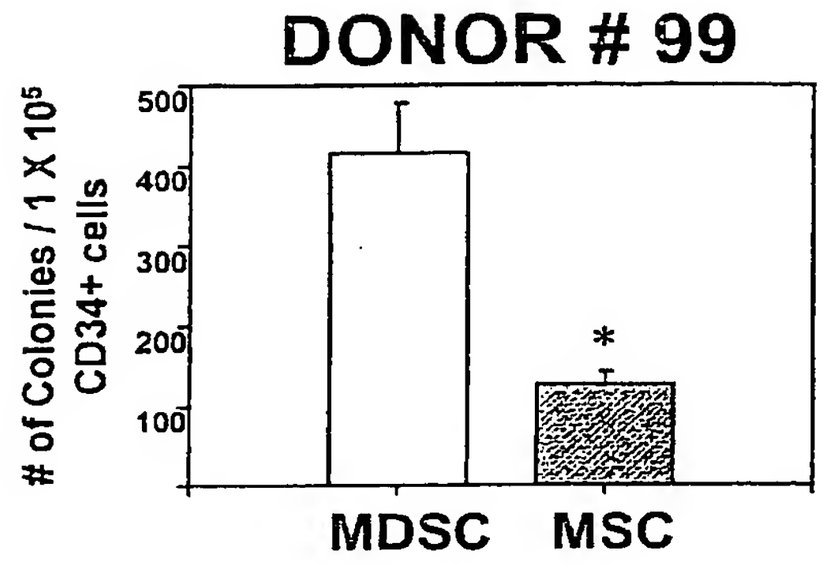
Cytokines/ growth factors	Transcript copies per nanogram of RNA	
	Untreated	IL-1α-treated
β-2 microglobulin	6,100	6,300
IL-1α	14	7,100
G-CSF	3	2,500
GM-CSF	18	1,500
LIF	8	2,800

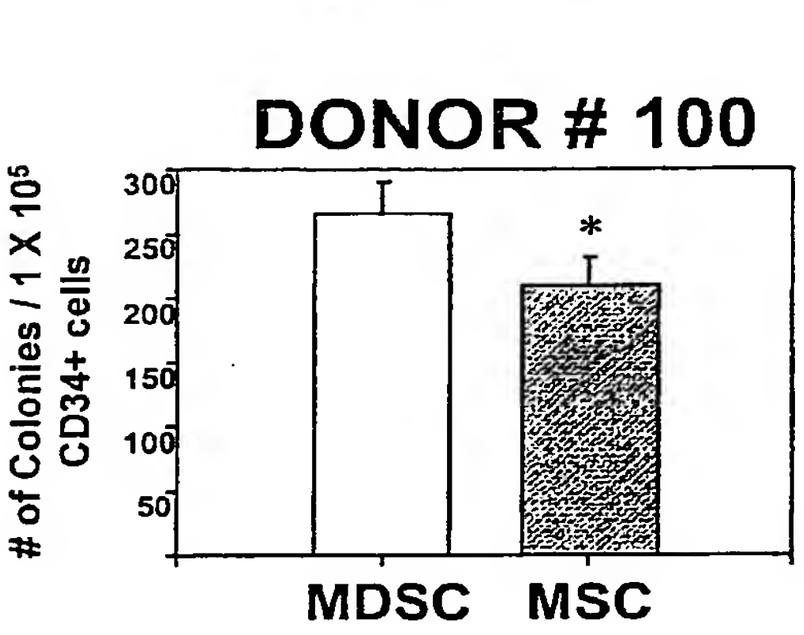
[1]Total RNA was prepared from MSCs untreated and treated with IL-1α for 24 h. RT-PCR was performed using total RNA and specific oligonucleotide primers for the cytokines and growth factors. Quantitation of PCR amplified products were performed using 5'-biotinylated oligonucleotide primer probes and the procedure for PCR-ELISA analysis. The number of gene copies for the cytokines and growth factors was calculated relative to the PCR amplification of known gene copies for the β-2 microglobulin gene.

nents and their function in hematopoiesis remain unclear. To this end, we developed methods for the isolation of pluripotent MSCs, an adherent bone marrow cell population that can be expanded in culture without differentiation (Haynesworth et al., 1992b). Here, we examined the morphology, phenotype, and function of MSCs in comparison with MDSCs, the standard cell preparation for in vitro stromal cell culture (Dexter et al., 1977). By the criteria used in this study, we established that culture-expanded MSCs are a homogenous population of fibroblastoid cells distinct from MDSCs which are comprised of both fibroblastic and hematopoietic cells. Like MDSCs, MSCs express numerous cytokines and growth factors and support hematopoiesis in LTBMC, suggesting that MSCs represent an important cellular and functional component of the the stroma.

Our data show that cultures of MSCs are distinct both in morphology and in cellular composition from cultures of MDSCs prepared from the same bone marrow cells. There are several possible explanations for the difference seen in the two cultures, including the methods established for isolation and culture expansion of the cells. First, MSCs are isolated using Percoll (1.073 g/ml) density sedimentation, while MDSCs were cultured following Ficoll-Paque (1.077 g/ml) fractionation. The small difference in the densities between the Percoll and the Ficoll-Paque solutions may be selective for distinct cell populations. The higher density Ficoll-Paque may result in the isolation of cells that sediment through the Percoll solution used for MSC isolation. We believe that the composition of the medium contributes substantially to the disparity of the morphology and composition of the cultures. MDSCs are cultured in LTBMC medium that contains 25% serum (12.5% FBS, 12.5% horse serum) and 1 μM hydrocortisone. In contrast, MSC medium consists of DMEM-LG plus 10% of an FBS selected for the optimal growth of these cells. It is likely that the combination of differences in the







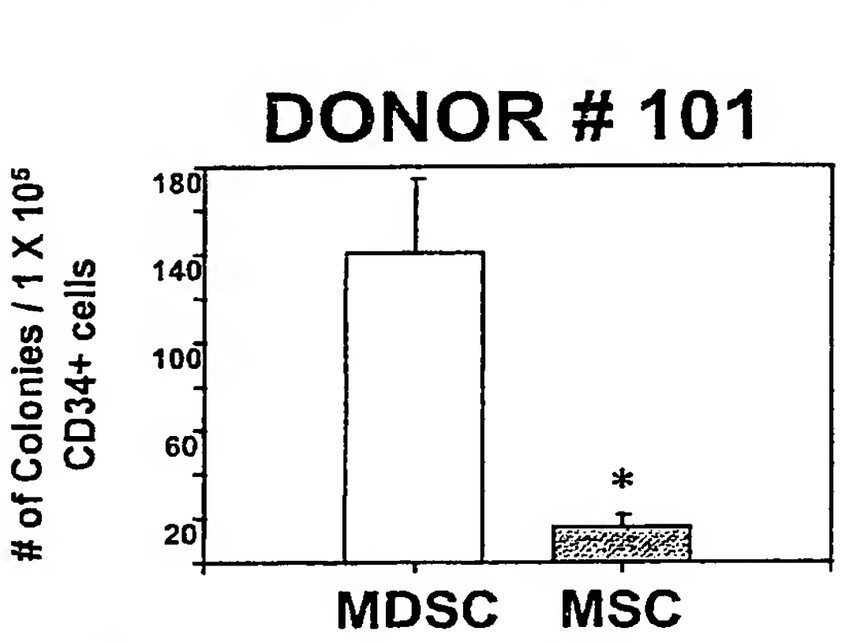


Fig. 4. Comparison of the ability to support hematopoiesis in vitro by MDSCs and MSCs from four donors. Passage 1 cultures of MDSCs and MSCs were irradiated and plated in six-well culture plates. $CD34^+$ cells were isolated from bone marrow and placed in coculture irradiated monolayers of MDSCs and MSCs. Both nonadherent and adherent cells were recovered and cultured in methylcellulose medium for 2 weeks, and hematopoietic colonies greater than 50 cells were counted. The bars represent the mean (±SEM) colony numbers from three individual wells. * Statistical significance ($P < .05$, Student's t-test) between MDSCs and MSCs.

isolation method and composition of the culture medium used in the preparation of MSCs and MDSCs contributes to the diverse cellular composition of these cultures.

The hallmark of MDSCs and other stromal cells is their capacity to produce a spectrum of cytokines and growth factors that regulate the proliferation, differentiation, and maintenance of HSCs and its precursors. Stromal cells also increase their synthesis of cytokines such as IL-1α and β, IL-6, G-CSF, and GM-CSF in response to treatment with IL-1α (Paul et al., 1990; Eaves et al., 1991; Sutherland et al., 1991; Cicuttini et al., 1992; Mosca et al., 1995). Haynesworth et al. (1996) previously used ELISA analysis to show that MSCs constitutively secrete IL-6, IL-11, LIF, SCF, and M-CSF and that IL-1α treatment increased the release of IL-6, IL-11, and LIF by MSCs and also induced the secretion of G-CSF and GM-CSF, which was undetectable in untreated cultures. In this study we not only observe the above results but additionally show that MSCs express mRNAs for other cytokines such as IL-7, IL-8, IL-12, IL-14, IL-15, and Flt-3 ligand. Our results also show that MDSCs produce a very similar array of cytokines and growth factors that act on hematopoietic cells. Since our analysis was not quantitative, little can be said about any differences observed in the constitutive levels of these mRNA in MSCs and MDSCs. Interestingly, IL-1α treatment caused an apparent induction of mRNA for IL-1α, IL-1β, and LIF only in cultured MSCs, suggesting distinct differences in the responsiveness of the two cell preparations. The biological relevance of IL-1 in the bone marrow microenvironment is best characterized in models of infection and inflammation. IL-1 mediates, in part, induction of circulating levels of colony stimulating factors, as shown by injecting IL-1 or by blocking IL-1R (Neta et al., 1990). There is also a well-described protective effect of IL-1 in mice after irradiation or cytotoxic drugs (Schwartz et al., 1987). From Figure 3, we observe that MSCs respond to IL-1α distinct from MDSCs especially by autocrine regulation of IL-1. The biological role of IL-1α is primarily as a regulator of intracellular events and a mediator of local inflammation, whereas IL-1β is a systemic, hormone-like extracellular mediator (Dinarello, 1996). Therefore, it is possible that, during inflammation and or infection, MSCs by their cytokines and growth factor expression may be able to influence protective reactions locally as well as at a distance from the bone marrow.

The LTBMC has been established as a standard assay to measure the function of stromal cell cultures in maintaining hematopoiesis in vitro (Dexter et al., 1977; Gartner and Kaplan, 1980). In this study we examined the ability of MSCs to support hematopoiesis in LTBMC and compared the ability of MSCs and MDSCs derived from the same marrow sample to maintain LTC-IC in coculture with $CD34^+$ hematopoietic progenitors. As anticipated, we observed cobblestone areas consisting of hematopoietic cells in cultures of both MSCs and MDSCs, suggesting the development of an in vitro marrow microenvironment. The results of methylcellulose hematopoietic colony assays showed that MSCs are able to maintain and support hematopoietic differentiation of purified $CD34^+$ cells. The results also showed that MSCs were not as efficient as MDSCs in maintaining hematopoiesis in vitro. The number of colonies arising from cocultures of $CD34^+$ cells with MSCs ranged from 11–100% of that for the corresponding MDSC preparation. A possible explanation for the difference in hematopoietic support between the two cultures may lie in the heterogenous nature of the MDSC cultures. It is also possible that other accessory cells like $CD14^+$ hematopoietic cells present in MDSCs may release molecules such as IL-1 and alter the steady-state production of various cytokines by cellular components present in MDSC cultures (Mielcarek et al., 1996). This cell-to-cell interplay may provide an advantage to the MDSC culture in the subsequent support of hematopoietic differentiation. Cultured MSCs should allow us to dissect out the role of such accessory cells starting from selected cell populations. It is also possible that MSCs from different donors are intrinsically different in their synthesis of hematopoietic cytokines, which may dictate the efficiency by which the cells can support hematopoiesis. It is clear from our studies that culture-expanded MSCs maintain LTC-IC in LTBMC and therefore represent an important cellular component of the bone marrow microenvironment.

Caplan (1991) previously postulated that the bone marrow is resident to a population of pluripotent cells capable of differentiating into various mesenchymal lineages. MSCs have been shown to differentiate along osteogenic (Haynesworth et al., 1992a), chondrogenic (Johnstone and Barry, 1996), adipogenic (Pittenger et al., 1996), tendonogenic (Young et al., 1997) lineages to demonstrate their pluripotential characteristic. The results of this study not only provide evidence that MSCs are an important cellular component of the bone marrow microenvironment but also add further to the pluripotent nature of MSCs. Previous work has shown that cultured osteoblastic cells can support hematopoiesis in vitro (Taichman and Emerson, 1994; Taichman et al., 1996), and there is recent data indicating a similar function of cultured adipocytes (Gimble et al., 1996). Therefore, it will be of great interest to investigate the link between lineage differentiation of the MSC and its capacity to maintain hematopoiesis in vitro. The ability to expand MSCs in culture without inducing differentiation makes the MSC an excellent candidate for preclinical and clinical applications in stromal cell therapies.

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